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(57) Abstract

Methods for determining the outcome of cytokine therapy in subjects suffering from chronic virus infections are provided, as well as methods for predicting the outcome of persistent virus infections, eg persistent HBV infections. Kits for use in such methods are also provided.

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METHODS FOR PREDICTING THE OUTCOME OF PERSISTENT HBV INFECTION AND THE OUTCOME OF CYTOKINE THERAPY

The present invention relates to methods of predicting the outcome of persistent HBV infection in a subject, as well as the outcome of cytokine therapy, particularly interferon therapy, in patients suffering from Chronic Hepatitis infection, particularly chronic HBV infection.

Approximately 300 million people worlwide are persistently infected with hepatitis B virus (HBV). In people exposed to the virus after the neonatal period the majority recover and only 5-10% will develop a persistent infection. Up to 50% of these are likely to die prematurely from end stage liver disease or hepatocellular carcinoma which are complications of HBV infection (Beasly *et al*, *J.Infect.Dis.*, 2:1129-33 (1981)). Viral elimination may be induced by treatment with interferon alpha or lymphoblastoid interferon. However, the response rate for this therapy is limited, eg only around 40% in the case of chronic HBV.

In addition, interferon therapy can also be unpleasant as the majority of patients suffer influenza-type symptoms. More severe side effects include suppression of bone marrow, abnormalities of thyroid function and endogenous depression. Interferon treatment is also expensive and thus there are pressures to rationalise the use of such treatment within the healthcare community.

Twin studies have established that the outcome of HBV infection, transient or persistent infection, is, in part, determined by genetic susceptibility (Lin *et al*, *Anticancer Research*, 9:737 (1989)). We have previously

shown that the MHC class II allele DRB1*1302 is associated with resistance to persistent infecton (Thursz *et al*, *N.Eng.J.Med.*, 3321065 (1995)) and the codon 52 variant of the mannose binding protein gene with susceptibility to persistent HBV infection (Thomas *et al*, *Lancet* (1996) in press).

There is a bi-allelic polymorphism of the promoter for the TNF alpha gene. The mutation is located at position -308 relative to the transcription initiation sequence of the TNF alpha gene (Mcguire, *et al*, *Nature*, **371**:508-510 (1994)). The rare TNF-2 allele is associated with higher constitutive and inducible transcription of the TNF alpha gene. The TNF-2 allele is found to be in linkage disequilibrium with the B8-DR3 haplotype in caucasian subjects. At position -308, the rarer allele, known as TNF2, is associated with increased TNF production (Wilson *et al*, *Eur.J.Immunol.*, **24(1)**:191-5 (1994)). This allele and the -238 variant whose functional significance is unknown, have both been associated with adverse outcomes from other infectious diseases such as *P.falciparum* malaria.

TNF inhibits the expression of HBV in HBV transgenic mice (Daniels *et al*, *Lancet*, **335**:875 (1990); Guilhot *et al*, *J.virol.*, **67(12)**:7444-9 (1993)) and TNF production is higher in patients with chronic HBV infection who respond successfully to interferon therapy. This phenomenon occurs towards the end of a period of treatment and is therefore not of use in identifying, prior to treatment, patients likely to respond to interferon.

Furthermore, a study of factors which determine the response to interferon in Caucasians with chronic HBV

infection, identified an association with the MHC haplotype A1-B8-DR3 (Scully,*et al*, *Hepatology*, **12**:1111-1117 (1990)) which is in strong linkage disequilibrium with the TNF2 allele in Caucasian populations.. Of 11 patients with this haplotype, 10 responded to interferon whilst only 1 did not. This one patient was subsequently found to have a lymphoma which may have impaired his immune function and thus impaired his ability to eradicate the virus even with the help of interferon.

There thus exists a clinical need for a method or predictive test which would allow assessment of subjects suffering from a chronic hepatitis virus infection which, in turn, would allow cytokine, eg interferon, treatment to be targeted to those patients with a reasonable chance of responding.

We have now found that all patients who carry the TNF-2 allele respond to interferon treatment. However, for patients homozygous for the TNF-1 allele it must be assumed that only about 40% will repond to interferon treatment.

Polymorphism of any cytokine or cytokine promoter, including Alpha interferon subtypes, gamma interferon, IL2, IL4, IL5, IL6, IL10 and IL12, could also be expected to influence outcome, not only of hepatitis B infection, but also hepatitis C, hepatitis G, human papilloma virus, human immunodeficiency virus and other persistent virus infections. Thus, sensible targeting of cytokine therapy should be possible.

We have also established that the presence of the TNF-2 allele is also itself linked with the development of

persistent HBV infection.

Thus, in a first aspect, the present invention provides a method for assessing and/or predicting the probable outcome of treating a subject suffering from a persistent virus infection with a cytokine comprising the step of determining whether the subject carries one or more alleles associated with an improved probability of a therapeutic response when treated with said cytokine.

In one embodiment of this aspect of the invention, the persistent virus infection is a hepatitis virus infection, particularly hepatitis B. In the case of the latter the method comprises determining whether the subject carries the TNF-2 allele.

In a second aspect the present invention provides a method for assessing and/or predicting the probable outcome of hepatitis virus infection in a subject comprising the step of determining whether the subject carries the TNF-2 allele.

In one embodiment of this aspect of the invention, the hepatitis virus infection is hepatitis B virus (HBV) infection, and in particular the method allows a determination of whether the subject is more likely to develop a persistent HBV infection.

The preferred method of carrying out the determination for both these aspects of the invention is to analyse a sample of the subject's DNA. Such a sample can conveniently be obtained from a biological sample, eg a blood sample.

Suitably, the DNA obtained from the biological sample will be amplified using techniques well known to those skilled in the art, eg PCR techniques. For example, the TNF alpha gene region, and more particularly, the TNF alpha promoter region, can be amplified. Such techniques will involve the use of at least one pair of suitable primers. Suitable primers can be chosen on the basis of the DNA sequence of the cytokine gene in question , eg the TNF alpha gene.

In the context of the present invention, TNF alpha gene region can mean the whole of the TNF alpha gene or, alternatively, a part thereof. Clearly, however, if only a part is amplified it should include the promoter for the gene since this is where the TNF-2 allele will be found , if it is present.

In a third aspect, therefore, the present invention provides a kit for use in a method for assessing and/or predicting the probable outcome of treating a subject suffering from a persistent virus infection with a cytokine, which comprises at least one pair of primers suitable for PCR amplification of at least a portion of the gene coding for the cytokine.

In a fourth aspect, therefore, the present invention provides a kit for use in a method for assessing and/or predicting the probable outcome of hepatitis virus infection in a subject, which comprises at least one pair of primers suitable for PCR amplification of at least a portion of the gene coding for the TNF alpha.

Preferred features of each aspect of the invention are as for each other aspect, *mutatis mutandis*.

The invention will now be described by reference to the following examples, which should not be construed as in any way limiting the invention.

Example 1

A group of 14 patients suffering from chronic hepatitis B virus infection were studied. All the patients had been undergoing treatment with either alpha interferon or lymphoblastoid interferon for at least three months.

Samples of peripheral blood were collected for each patient and DNA extracted using a Nucleon II kit (Scott labs). DNA was apmplified using PCR and TNF alpha promoter alleles were detected using dot blot hybridisation using the method described by McGuire et *al*, *supra*.

Of the 14 patients, 11 were responding to the interferon treatment. Of these 11, 5 were homozygote for the common TNF-1 allele and 6 were either heterozygote or homozygote for the TNF-2 allele. Of the three patients who failed to respond to the treatment, none carried the TNF-2 allele (ie they were all homozygous for the TNF-1 allele).

Thus, it would appear that the TNF-2 allele confers an increased probability of response to interferon.

Example 2

A large case-control study was performed on a population in the Gambia. Participants were all west Africans living in the western coastal region of the Gambia in the area surrounding the capital, Banjul. Two different groups were recruited for the study between 1988 and 1990. In the first group, children up to 10 years were recruited

at the Royal Victoria Hospital, Banjul and the Medical Research Council (MRC) Hospital, Fajara where they had been seen for a variety of conditions unrelated to HBV. The second group were adult healthy male blood donors. Both populations had previously been studied as part of a case-control study of susceptibility to malaria. Ethical permission for this study was granted by the MRC Gambian Government joint Ethics Committee. Consent for phlebotomy was obtained from each child's parent or guardian.

Participants were divided into groups according to the result of serological tests for HBV. People who had never been exposed to HBV, were HBV core antibody (anti-HBc) negative. People who had spontaneously recovered from HBV infection were IgG anti-HBc positive and HBV surface antigen (HBsAg) negative. People with persistent HBV infection were IgG anti-HBc and HBsAg positive. People with acute HBV infection, who may not have had sufficient opportunity to eliminate HBsAg (HBsAg positive and IgM anti-HBc positive) were excluded from the analysis. 107 people who had been vaccinated against HBV (<10%) were classified in the uninfected group and were therefore not included in the analysis of the TNF allele frequencies. The five individuals with HIV antibodies (<1%) were also excluded.

Serological Testing

Plasma samples were taken from all participants and stored at -20°C. Anti-HBc (total), anti-HBc (IgM), HBsAg status and anti-HBs concentration were dtermined by ELISA according to the manufacturers instructions (Boehringer Mannheim, Munich, Germany). HIV antibodies were dtermined by Wellcozyme ELISA (Wellcome, Beckenham, UK). Positive

results were confirmed by western blot.

TNF promoter -308 variant genotyping

The polymerase chain reaction was used to amplify a 519 base pair fragment of the promoter from position -502 to +17. The primer sequences were:

5'-CAAACACAGGCCTCAGGACTC-3'

3'-AGGGAGCGTCTGCTGGCTG-5'

About 100ng (2μl) of genomic DNA which had been extracted from peropheral blood was added to 25μl of reaction mix which contained 0.1μM of each primer. 100μM of each dNTP, 67mM tris-HCl, 16mM $(NH_4)_2SO_4$, 2mM $MgCl_2$ and 0.01% Tween-20.

The reaction mix was heated to 95°C for 10 minutes then 1.5U Taq DNA polymerase was added. The reaction mix was then given 35 cycles of 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute in a Perkin-Elmer 9600 Thermocycler with heated lid. The PCR product was finally left at 72°C for 10 minutes.

The PCR product was denatured with 0.4M NaOH, dotted on to nylon membranes (Amersham High-Bond N) using a vacuum manifold and fixed for 1 minute with UV light. Allele-specific oligonucleotides were end labelled with digoxigenin-ddUTP and hybridized to the membrane with 3M TMAC (tetramethylammonium chloride) solution at 55°C for 1 hour. Excess probe was removed by washing in 2 x SSPE/0.1% SDS at room temperature and subsequently in 3M TMAC at 58°C. The membranes were treated with anti-digoxigenin Fab fragments and, after washing to remove excess antibody, they were incubated with Lumigen PPD and exposed to X-ray film for 15-30 minutes. Allele type was scored by two independent observers. The accuracy of this

method was confirmed by DNA sequencing.

-308 variant oligonucleotide probes:
wild type 5'-AGGGGCATGGGGACGGG-3'
-308 variant 5'-AGGGGCATGAGGACGGG-3'

TNFα promoter -238 variant genotyping

The -238g allele was typed by amplification refractory mutation system PCR using a conserved primer (5'-AAGACCCCCCTCGGAATCA-3') together with sequence specific primers for either the -238g allele (5'-AGACCCCCCTCGGAATCG-3') or the 238a allele (5'-AAGACCCCCCTCGGAATCA-3') to generate a 459bp product. As a positive control each reaction mixture contained the following primers:
5'-CCAAAGATTCAGGTTTACTCACG-3'; and
5'-ACTTAACTATCTTGGGCTGTGAC-3'
to amplify a 266bp fragment of the human α2 microglobulin gene. Negative controls, using water instead of template, were included on each plate. Each 25μl reaction mixture contained 100ng genomic DNA, 67mM Tris HCl, 16mM ammonium sulphate, 2mM magnesium chloride, 100μM of each dNTP, 0.5 units of Taq polymerase (Bioline), 0.1μM of each TNF primer and 0.2μM of each α2 microglobulin primer. The mixture was incubated at 95°C for 5 minutes followed by 5 cycles of (1 minute at 95°C, 1 minute at 67°C, 1 minute at 72°C), then 25 cycles of (1 minute at 95°C, 1 minute at 62°C, 1 minute at 72°C) and finally 10 minutes at 72°C. The products were resolved on a 2% agarose gel stained with ethidium bromide and visualised under UV light.

Statistical analysis

Univariate analysis was performed by comparing the proportion of subjects with each allelic variant between cases (HBsAg positive) and controls (HBsAg negative) using the crude odds ratio, exact 95% confidence intervals and p values calculated using the χ^2 test. Multivariate analysis was performed using a logistic regression model to allow for the interaction of the TNF alleles with HLA class II alleles and with other possible confounding variables such as age, sex, ares of residence and ethnic background. each term was added to the model in a stepwise method and selected for inclusion when it resulted in a significant improvement of the model with a statistical significance of $p>0.05$.

Results

Position -308 variants

Table 1: Allele frequencies for the TNFα promoter -308 variants in subjects with persistent HBV (HBsAg positive anti-HBc (igM) negative) and with self limiting HBV infection (anti-HBc (total) positive, HBsAg negative).

	-308 Heterozygotes (%)	-308 Homozygotes (%)	Sample Size
Self limiting infection	24.5	3.6	332
Persistent HBV infection	32.7	3.5	171
HBeAg positive	34.3	4.0	99
HBeAg negative	36.1	2.7	36

In the persistently infected group there were 6 (3.5%) homozygotes for TNF2 and 56 (32.7%) heterozygotes, compared to 12 (3.6%) homozygotes and 80 (24.1%) heterozygotes in the transiently infected group. In analysing these data it was necessary to stratify for HLA DRB1*1302, which is associated with HBV clearance and is in linkage disequilibrium with the TNF2 allele in this population. After stratification, the odds ratio for persistent HBV infection in subjects with the TNF2 allele was 1.56 (95% confidence interval 1.03-2.38). When the children with severe malaria (associated with TNF2) are excluded from the analysis the odds ratio for persistent

infection for subjects with TNF2 is 2.03 (95% C.I. 1.02-4.05), p=0.04. In the logistic regression model, terms were included for TNFα -308 genotype, ethnic background, area of residence, the presence of cerebral malaria, the presence of DRB1*1302 and the presence of HLA-DQ8. The variables TNFα -238 genotype, age and sex were rejected because they did not result in a significant improvement in the model. Using this model the associateion of TNF2 with persistent HBV infection was confirmed with p=0.032.

Position -238 variants

Table 2: Allele frequencies for the TNFα promoter -238 variants in subjects with persistent HBV (HBsAg positive anti-HBc (IgM) negative) and with self limiting HBV infection (anti-HBc (total) positive, HBsAg negative).

	-238 Heterozygotes (%)	-238 Homozygotes (%)	Sample Size
Self limiting infection	12.0	1.1	184
Persistent HBV infection	9.8	3.3	61
HBeAg positive	9.6	3.2	31
HBeAg negative	0	0	5

The frequency of these alleles were similar in the transient and persistently infected subjects.

Discussion

These results demonstrate that the -308 allelic variant (TNF2) but not the -238 variant, is associated with incresed susceptibility to persistent HBV infection with a relative risk of approximately 2. This finding is at first sight surprising as previous studies have indicated that TNF has antiviral properties and it would be anticipated that alleles associated with increased TNF production (TNF2) would be associated with enhanced viral clearance. However, a possible explanation can be found if recent observations that activation induced cell death in mature lymphocytes can be mediated through the TNF receptor as well as through the better known apoptosis receptor, Fas are taken into account. These studies describe inhibition of virus-specific CD8+ T cell expansion mediated by TNF suggesting that disregulation of TNF production may actually produce tolerance to viral antigens (Zheng *et al*, *Nature*, **377**:348-51 (1995)). These in-vitro phenomena may contribute to the failure of patients with persistent HBV infection to mount an adequate CD8+ cytolytic response comparable to that observed in acute infection followed by recovery (Bertoletti *et al*, *PNAS USA*, **88(23)**:10445-9 (1991)).

CLAIMS:

1. A method for assessing and/or predicting the probable outcome of treating a subject suffering from a persistent virus infection with a cytokine comprising the step of determining whether the subject carries one or more alleles associated with an improved probability of a therapeutic response when treated with said cytokine.

2. A method for assessing and/or predicting the probable outcome of hepatitis virus infection in a subject comprising the step of determining whether the subject carries the TNF-2 allele.

3. A method as claimed in claim 1 wherein the persistent virus infection is hepatitis B infection, hepatitis C infection, hepatitis G infection, human papilloma virus infection or human immunodeficiency virus infection.

4. A method as claimed in claim 3 wherein the persistent virus infection is a hepatitis virus infection.

5. A method as claimed in claim 4 wherein the hepatitis virus infection is a chronic hepatitis B virus infection.

6. A method as claimed in any one of claims 1 to 5 wherein the cytokine is lymphoblastoid interferon, Alpha interferon, including subtypes thereof, gamma interferon, IL2, IL4, IL5, IL6, IL10 or IL12.

7. A method as claimed in claim 6 wherein the cytokine is lymphoblastoid interferon or Alpha interferon.

8. A method as claimed in claim 7 wherein it is determined whether the subject carries the TNF-2 allele.

9. A method as claimed in claim 2 wherein the hepatitis virus infection is a hepatitis B (HBV) virus infection.

10. A method as claimed in claim 9 wherein the method is for determining whether the subject is more likely to develop a persistent HBV infection.

11. A method as claimed in any one of claims 1 to 10 wherein the determination is carried out using DNA obtained from a biological sample.

12. A method as claimed in claim 11 wherein the biological sample is a blood sample.

13. A kit for use in a method for assessing and/or predicting the probable outcome of treating a subject suffering from a persistent virus infection with a cytokine, which comprises at least one pair of primers suitable for PCR amplification of at least a portion of the gene coding for the cytokine.

14. A kit as claimed in claim 13 modified by any one or more of the features of any one or more of claims 3 to 8.

15. A kit for use in a method for assessing and/or predicting the probable outcome of hepatitis virus infection in a subject, which comprises at least one pair of primers suitable for PCR amplification of at least a portion of the gene coding for the TNF alpha.

16. A kit as claimed in claim 15 modified by any one or

more of the features of claim 9 or claim 10.

17. A kit as claimed in any one of claims 13 to 16 which includes one or more of the following pairs of primers:

5'-CAAACACAGGCCTCAGGACTC-3'
3'-AGGGAGCGTCTGCTGGCTG-5';

5'-AAGACCCCCCTCGGAATCA-3' with either
i) 5'-AGACCCCCCTCGGAATCG-3' or
ii) 5'-AAGACCCCCCTCGGAATCA-3'; or

5'-CCAAAGATTCAGGTTTACTCACG-3'
5'-ACTTAACTATCTTGGGCTGTGAC-3'.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 332, no. 16, April 1995, pages 1065-69, XP000614925 THURSZ M ET AL: "Association between an MHC class II allele and clearence of Hepatitis B Virus in the Gambia" cited in the application see the whole document ---	1
A	THE LANCET, vol. 335, 14 April 1990, pages 875-77, XP002024125 DANIELS H ET AL: "Spontaneous production of tumour necrosis factor alpha and interleukin-alpha during interferon treatment of chronic HBV infection " cited in the application see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol. 371, 6 October 1994, pages 508-11, XP002024126 MCGUIRE W ET AL: "Variation in the tnf-alpha promoter region associated with susceptability to cerebral malaria" cited in the application see the whole document	13-17
A	ALIMENTARY PHARMACOLOGY AND THERAPEUTICS, vol. 8, no. 2, April 1994, pages 229-53, XP000616907 DUSHEIKO G: "Rolling review- the pathogenesis, diagnosis and management of viral hepatitis" see page 245, paragraph 4 - page 246, paragraph 2	1
A	HEPATOLOGY, vol. 12, no. 5, November 1990, pages 1111-17, XP000614963 SCULLY L ET AL: "Immunological studies before and during interferon therapy in chronic HBV infection: identification of factors predicting response" see the whole document	1
A	NATURE MEDICINE, vol. 1, no. 4, April 1995, pages 374-5, XP000616344 THURSZ M ET AL: "Association of hepatitis B surface antigen carriage with severe malaria in Gambian children" see paragraph 1	1

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